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Interleukin-6 family of cytokines induced activation of different functional sites expressed by gp130 transducing protein.

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Gp130 transducing protein was shown to be involved in the formation of the high affinity receptors for interleukin 6 (IL-6), interleukin-11 (IL-11), leukemia inhibitory factor, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1. In the present study we have characterized the functional properties of antibodies directed against this protein and identified a group of monoclonal antibodies able to antagonize the biological activities of all the cytokines belonging to the IL-6 cytokine family. The B-R3 pan-blocking antibody weakly interfered with the binding of the radiolabeled ligands (with the exception of OSM, whose binding was abrogated in the presence of B-R3 monoclonal antibody) but inhibited the gp130 homodimerization or its association with gp190/leukemia inhibitory factor receptor, as well as the subsequent tyrosine phosphorylation events. In addition we identified antibodies that were able to neutralize only one single cytokine of the IL-6 family. This was the case for the B-K5 antibody, which antagonized the binding of OSM to gp130 but did not interfere with the signals provided by the related cytokines triggering the proliferation of the TF1 erythroleukemia cell line or the induction of haptoglobin synthesis in the HepG2 hepatoma cell line. Similarly, we also characterized two additional antibodies B-P8 and B-P4, which inhibited the TF1 cell proliferation observed in the presence of CNTF and IL-11, respectively. B-P8 antibody only faintly interfered with the binding of the gp130-ligands and might modulate the signal transduction pathways. This study indicates that in addition to functional site(s) required by the whole family of IL-6 type cytokines to transduce the signal insight the cell, specific cognate functional sites were recruited by OSM, CNTF, or IL-11.

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Interleukin-6 Family of Cytokines Induced Activation of Different Functional Sites Expressed by gp130 Transducing Protein*

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Gp130 transducing protein was shown to be involved in the formation of the high affinity receptors for interleukin 6 (IL-6), interleukin-11 (IL-11), leukemia inhibitory factor, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1. In the present study we have characterized the functional properties of antibodies directed against this protein and identified a group of monoclonal antibodies able to antagonize the biological activities of all the cytokines belonging to the IL-6 cytokine family. The B-R3 pan-blocking antibody weakly interfered with the binding of the radiolabeled ligands (with the exception of OSM, whose binding was abrogated in the presence of B-R3 monoclonal antibody) but inhibited the gp130 homodimerization or its association with gp190/leukemia inhibitory factor receptor, as well as the subsequent tyrosine phosphorylation events. In addition we identified antibodies that were able to neutralize only one single cytokine of the IL-6 family. This was the case for the B-K5 antibody, which antagonized the binding of OSM to gp130 but did not interfere with the signals provided by the related cytokines triggering the proliferation of the TF1 erythroleukemia cell line or the induction of haptoglobin synthesis in the HepG2 hepatoma cell line. Similarly, we also characterized two additional antibodies B-P8 and B-P4, which inhibited the TF1 cell proliferation observed in the presence of CNTF and IL-11, respectively. B-P8 antibody only faintly interfered with the binding of the gp130-ligands and might modulate the signal transduction pathways. This study indicates that in addition to functional site(s) required by the whole family of IL-6 type cytokines to transduce the signal insight the cell, specific cognate functional sites were recruited by OSM, CNTF, or IL-11.

related cytokines share many common biological properties, such as activation of hepatocyte transcription (3), activation of neural proliferation and differentiation (4), and regulation of hematopoiesis (5, 6). In addition LIF, CNTF, CT-1, and OSM display biological properties in the early stages of embryonic development and allow the *in vitro* growth of embryonic stem cells in an undifferentiated state (7-9). IL-6 and IL-11 are also important modulators for the immune response by regulating immunoglobulin secretion (10, 11). The redundancy of their biological properties is in part explained by the shared use of the common signaling protein, gp130, in their multimeric receptors. Gp130, initially isolated as an IL-6 signal transducer (12), associates with other receptor components to generate high affinity type receptors for the ligands. This is the case for the gp190/low affinity LIF receptor (13), which associates with gp130 to generate a functional LIF/OSM/CT-1 receptor (9, 14). For the CNTF receptor a third component (α CNTF receptor subunit (15)) interacts with the gp130/gp190 heterocomplex to generate a high affinity CNTF receptor (16). The IL-6 receptor is composed of a gp130 homodimer associated with an IL-6 binding chain, gp80 (17). Recently a binding subunit for IL-11 was also isolated (18), and an additional specific OSM type II receptor was described (19).

Dimerization of the transducing subunits initiates intracellular signaling by activating members of cytokine receptor-associated tyrosine kinases, referred to as Jaks (for review see Ref. 20). Both gp130 and gp190/LIF receptor can associate Jak1, Jak2, and Tyk2 (21). The information is next relayed by a family of transcription factors known as STATs (signal transducers and activators of transcription), which are activated in the cytoplasm before translocation to the nucleus (20). IL-6-related cytokines will preferentially activate STAT1 and STAT3 (22-24).

In the present study we have analyzed a new set of monoclonal antibodies raised against gp130 and tried to identify functional sites at the surface of the gp130 transducing protein. This work should help to understand the level of specificity brought by one designed cytokine activating this pathway.

MATERIALS AND METHODS

Cells and Reagents—The SK-N-MC neuroblastoma cell line (ATCC, Rockville, MD), the A375 melanoma cell line (ATCC) (25), and the HepG2 cell line (ATCC) (26) were routinely grown in RPMI culture medium supplemented with 10% fetal calf serum. For the growth of the multifactor-dependent erythroleukemia TF1 cell line (27), the culture medium was supplemented with 1 ng/ml GM-CSF. Purified human recombinant LIF (10^6 units/mg) produced in Chinese hamster ovary cell line, *Escherichia coli* recombinant GM-CSF (10^6 units/mg), and IL-11 (2.5×10^6 units/mg) were kindly donated by Dr. K. Turner (Genetics Institute, Boston, MA). Rat CNTF (2×10^7 units/mg) and the soluble human CNTF receptor, both expressed in *E. coli*, were obtained from Dr. C. D. Yancopoulos (Regeneron, Tarrytown, NY), whereas human IL-4 derived from *E. coli* (4×10^7 units/mg) was from DNAX Research Institute (Palo Alto, CA). CT-1 (2.5×10^6 units/mg) was a kind gift of Dr. D. Pennica

Interleukin-6 (IL-6)¹ belongs to an increasing family of cytokines (1), which includes leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), interleukin-11 (IL-11), and cardiotrophin-1 (CT-1) (2). These closely

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¹ The abbreviations used are: IL, interleukin; LIF, leukemia inhibitory factor; OSM, oncostatin M; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; GM-CSF, granulocyte macrophage colony-stimulating factor; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; ³H-Tdr, [³H]thymidine.

(Genentech, South San Francisco, CA). IL-6 (10^7 units/mg) and OSM (10^6 units/mg) were purchased from Peprtech (Canton, MA). For the antibody production, Balb/c mice were immunized with the soluble form of gp130 (R & D Systems, Minneapolis, MN). After three injections the animals were boosted with 2 μ g of gp130, and the spleen cells fused 4 days later with the X63 myeloma cell line. Hybridoma supernatants were screened 10 days later by cytofluorometry on a Facscan (Becton Dickinson, Mountain View, CA) by using a BAF/Bo3 cell line stably transfected with a cDNA encoding for human gp130. After isotype typing, ascitic fluids were produced, and the immunoglobulins were protein A purified according to the usual protocols. With the exception of B-R3 and B-S8, which were IgG2a, all the remaining studied antibodies were IgG1.

Bioassays—The TF1 erythroleukemia cell line was plated at a concentration of 15×10^3 cells per well in RPMI containing 10% fetal calf serum and a fixed concentration of IL-6 (20 ng/ml), IL-11 (100 ng/ml), OSM (10 ng/ml), LIF (50 ng/ml), CT-1 (5 ng/ml), or CNTF (20 ng/ml) plus soluble CNTF receptor (100 ng/ml) as described previously (28, 32). Serial dilutions of antibodies were added in triplicate in the assay, and the cells were incubated for 72 h. 0.5 μ Ci of 3 HTdr were added in each well for the last 4 h of the culture, and the incorporated radioactivity was determined. HepG2 hepatoma cell line was plated in 96-well plates at a concentration of 50×10^3 cells/well in 200 μ l of RPMI medium containing 10% fetal calf serum, 10^{-6} M dexamethasone, and a fixed concentration of OSM (20 ng/ml) or IL-6 (10 ng/ml). Serial dilutions of antibodies were added in triplicate in the culture. After 48 h the supernatants were harvested, and the haptoglobin content was determined by ELISA. Symetric haptoglobin ELISA was developed as previously reported for human LIF detection (29). Briefly, purified goat immune IgG directed against human haptoglobin (A030 from Dako, Glostrup, Denmark) were coated overnight at a concentration of 2 μ g/ml in 100 mM carbonate buffer, pH 8.6. After washing and a saturation step with 100 mM Tris, 20% sucrose, pH 7.8, the samples were added to the wells in duplicate at a 1:10 and 1:50 dilution for a 6-h incubation at 37 °C. The same polyclonal antibody was biotinylated with NHS-LC-biotin (Pierce) and used as tracer antibody at a final concentration of 0.2 μ g/ml. After an overnight incubation, streptavidin peroxidase (Dako) was added at a 1:20,000 dilution for one additional hour incubation step. 2,2'-Azinobis (3-ethylbenzthiazolinesulfonic acid) (Sigma, l'Isle d'Abeau Chesnes, France) was used as substrate, and the reading was achieved at 405 nm. ELISA calibration was performed by using both calibrated standard serum from Behring (Rueil-Malmaison, France) and purified haptoglobin (Sigma). The haptoglobin detection limit in the ELISA was 20 pg/ml. A375 meloma cells were plated at a density of 3×10^3 cells per well in RPMI containing 1% fetal calf serum and 20 ng/ml OSM. Serial dilutions of antibodies were added in triplicate, and the cells were incubated at 37 °C, 5% CO₂ for 96 h. 0.5 μ Ci of 3 HTdr were added in each well for the last 4 h of the culture. After a freezing/thawing cycle to detach the cells from the plastic, the incorporated radioactivity was determined.

Radiolabeling and Binding Experiments—Purified recombinant LIF and OSM were iodinated by the two-phase method (30) as described previously (31). Briefly, 1–3 μ g of cytokine were incubated in a polypropylene tube (that had been cut off about 1 cm from the bottom) with 1 mCi of Na¹²⁵I. A filter paper previously soaked in 1 M NaCl and dried was placed on a coverslip. The reaction was initiated by spotting the filter with 10 μ l of chloramine T solution (16 mg/ml in 100 mM borate buffer, pH 8.5) and immediately inverting the coverslip and filter and placing them over the top of the reaction tube. The filter was changed twice over a 30-min incubation period. Excess iodine was separated from labeled protein by passage through a G-25 gel filtration column and equilibrated in 100 mM phosphate buffer containing 0.02% Tween 20. The specific activity was 10,000–20,000 cpm/fmol for LIF and 20,000–40,000 cpm/fmol for OSM. Radiolabeling of CNTF was performed by using the Enzymobead reagent (Bio-Rad). 1–3 μ g of CNTF in 0.1 M phosphate buffer were incubated in a total volume of 100 μ l containing 1 mCi of Na¹²⁵I, 0.25% glucose (w/v), and 50 μ l of hydrated beads. After a 30-min incubation period, excess unreacted iodine was removed as described above for LIF or OSM. The specific activity was 6,000–9,000 cpm/fmol. Radiolabeled IL-6 (3,500 cpm/fmol) was bought from Amersham Corp. Cells ($120\text{--}200 \times 10^6$ /ml) were incubated at 4 °C under gentle agitation in phosphate-buffered saline containing 0.5% bovine serum albumin, 20 mM Hepes, and 666 nM of the studied antibody or a control IgG. After 1 h, 25- μ l aliquots ($3\text{--}5 \times 10^6$ cells) were added to an equivalent volume of phosphate-buffered saline, 0.5% bovine serum albumin, 20 mM Hepes containing serial 0.66-fold dilutions of radiolabeled ligand. The highest final radiolabeled ligand concentration used was 1 nM. Nonspecific binding was evaluated by including a 100-fold excess of unlabeled cytokine. After a 2-h incubation period at

4 °C, cell-bound radioactivity was separated from the unbound fraction by centrifugation through a layer of 90% dibutylphthalate, 10% paraffin oil. Determination of affinity binding components was performed by nonlinear regression analysis of the data according to Scatchard.

Tyrosine Phosphorylation Analysis—SK-N-MC cells were starved in RPMI alone for 5–7 h (28). The antibodies were added to the plates at a final concentration of 50 μ g/ml for 1 h at 37 °C, 5% CO₂. The cells were then stimulated for 10 min at 37 °C in the presence of 50 ng/ml of the indicated cytokine, before being lysed in 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, and proteinase inhibitors (1 μ g/ml pepstatin A, 2 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). To coprecipitate gp190/LIF receptor with gp130, 1% Brij96 was used instead of Triton X-100. Cell lysates were rotated at 4 °C for 60 min, and insoluble material was pelleted at 12,000 \times g for 30 min. After protein standardization, the supernatants were immunoprecipitated overnight in the presence of either B-T2 or B-T6 antibody raised against gp130 at a concentration of 10 μ g mAb/ml. The complexes were then isolated with beads coupled to protein A, washed in lysis buffer, and eluted off the beads by boiling under reducing conditions for 5 min before being run on a 7.5% acrylamide SDS-polyacrylamide gel electrophoresis and transferred onto an Immobilon membrane (Millipore, Bedford, MA). After blocking the membrane was incubated with an anti-phosphotyrosine monoclonal antibody (4G10) from UBI (Lake, Placid, NY) at a final concentration of 1 μ g/ml, followed by an incubation with a goat anti-mouse immunoglobulin polyclonal antibody labeled with peroxidase at a 1:4,000 dilution (AMI 3404) (Tago, Camarillo, CA). For the detection of gp190/LIF receptor, the membranes were stained with a rabbit polyclonal antibody directed against LIF receptor (SC-659) (Santa Cruz Biotechnology, Santa Cruz, CA) used at a 1:500 dilution, followed by an incubation with biotinylated goat IgG recognizing rabbit immunoglobulins (1/1, 250) (E432) (Dako, Trappes, France). The membranes were then incubated in the presence of horseradish peroxidase conjugated to streptavidin (P397) (Dako) at a 1:5,000 dilution. The reactions were visualized on a x-ray film with the ECL reagent (Amersham Corp.) following the manufacturer's instructions.

RESULTS

Biological Properties of anti-gp130 mAbs—Gp130 transducing protein was shown to be involved in the formation of the high affinity receptors for IL-6, IL-11, LIF, OSM, CNTF, and CT-1 (1). In order to analyze the gp130 activation pathways in response to the different ligands, a series of monoclonal antibodies raised against this structure was generated and analyzed. The antibodies were first studied in several bioassays known to be sensitive to the members of the IL-6 cytokine family (25, 26, 28, 32). Analyses of the mAb blocking potentials in the proliferative response of the TF1 erythroleukemia cell line are represented in Fig. 1. Some of the antibodies such the B-R3, B-S8, or B-R12 were found to inhibit all the observed responses independently of the nature of the cytokine implicated in cell activation. Fig. 2A displays the curve responses obtained with the B-R3 antibody in the TF1 assay. Beside the group of pan-blocking antibodies, the TF1 cells allowed the discrimination of antibodies that only affected the response to one particular cytokine of the family without any effect on observed responses to the other cytokines. This was observed for B-S1, B-K11, and B-K5 antibodies, which blocked in a specific manner the OSM-triggered proliferation of the factor-dependent cell line (Figs. 1 and 2B). In addition, the B-P4 antibody specifically inhibited the TF1 proliferation to IL-11, whereas B-P8 antagonized the response observed in the presence of CNTF only. Fig. 3 illustrates the results obtained by adding increasing concentrations of the B-P4 or B-P8 antibodies in the assay. (Interestingly the B-P4 and B-P8 antibodies, which respectively interfered with the responses to IL-11 and CNTF, could displace each other in cross-competition experiments, indicating that they likely recognized closed epitopes on the gp130 molecule (data not shown).) We did not identify antibodies inhibiting LIF, CT-1, or IL-6 in a specific manner.

The biological properties of the gp130 antibodies were fur-

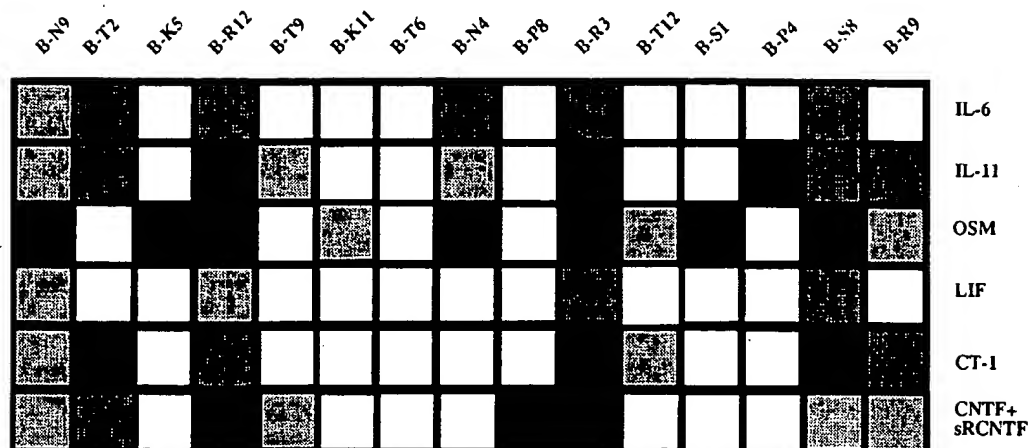


FIG. 1. Neutralizing activity of gp130 antibodies in the TF1 cell line proliferative assay. 15×10^3 TF1 cells were incubated in the presence of a fixed concentration of IL-6 (20 ng/ml), IL-11 (100 ng/ml), OSM (10 ng/ml), LIF (50 ng/ml), CT-1 (5 ng/ml), or CNTF (20 ng/ml) plus soluble CNTF receptor (100 ng/ml). Serial dilutions of antibodies were added in triplicate in the assay. After a 72-h culture period, ^3H Tdr was added for 4 h, and the incorporated radioactivity was determined. The figure summarizes a 50% inhibition of proliferation observed in the presence of 50 $\mu\text{g}/\text{ml}$ of the indicated antibodies (light gray), 10 $\mu\text{g}/\text{ml}$ (dark gray), 2 $\mu\text{g}/\text{ml}$ (black) or the absence of blocking effect observed in the presence of 50 $\mu\text{g}/\text{ml}$ of antibody (white).

ther characterized by analyzing their blocking potential in the induction of haptoglobin secretion in the HepG2 hepatoma cell line. In addition to gp130, the HepG2 cell line is known to express IL-6 receptor and both type I and II OSM receptors (19, 33). Introduction of increasing concentrations of antibodies in the presence of saturating amounts of OSM or IL-6 led to the results presented in Fig. 4A. Again, the B-R3 antibody was found to strongly antagonize the haptoglobin secretion induced by either OSM or IL-6. The B-K5 antibody was able to block the haptoglobin secretion only induced by OSM, confirming the result obtained in the TF1 cell line.

Because B-K5 mAb could antagonize OSM type I-mediated response in the TF1 cell line as well as OSM activation of HepG2 cell line expressing both types of OSM receptors, we decided to analyze the biological properties of the antibodies toward the A375 melanoma cell line, reported to only express type II OSM receptor (25). Cytotoxic activity of OSM was monitored by measuring the ^3H Tdr uptake in the A375 melanoma cell line cultured in the presence of anti-gp130 antibodies. Fig. 4B shows that both B-R3 and B-K5 were able to inhibit the cytostatic activity of OSM mediated through its type II receptor as well.

Interactions of gp130 mAbs on the Binding of the Ligands—In order to dissect with more details the blocking pathways of the pan-blocking antibody B-R3 and of the B-K5, B-P4, and B-P8 antibodies specifically interfering with OSM, IL-11, and CNTF, respectively, we have analyzed their interactions on the binding of the ligands to their receptors. To perform these experiments the ligands were radiolabeled, with the exception of IL-11, which turned out to be particularly unstable after radiolabeling.² Because the TF1 cell line expressed a very limited number of high affinity receptors for the IL-6-related ligands, the experiments were performed on the SK-N-MC human neuroblastoma cell line that we found to express reasonable amount of high affinity receptors for LIF, CNTF, IL-6, and OSM type I receptor. Saturation experiments were performed in the presence or the absence of a 666-fold molar excess of the studied antibodies (with the respect of two functional sites per antibody molecule). Scatchard transformation of the obtained values are presented in Table I. The results indicate that with the exception of the OSM, where a complete

abrogation of the cytokine binding to its receptor was observed, the B-R3 antibody only slightly affected the binding of the ligands for their receptors by a 2–5-fold decrease of the K_d value. The detected site number expressed at the cell surface remained unchanged, with again the exception of OSM. The nonblocking antibody B-T6 (see Fig. 1) used as IgG control did not interfere with the fixation of the radiolabeled cytokines. The addition of a 666-fold molar excess of B-K5 antibody in the saturation experiments completely abrogated the specific binding of OSM to its receptor. The binding inhibition was specific because the B-K5 antibody only faintly interferes with the binding of the other ligands to their receptors. Because it was previously reported that OSM directly contacted to gp130 when expressed alone (34), our results suggest that B-K5 recognized a major binding site for OSM on gp130. CNTF blocking antibody was also studied, and in contrast to the situation observed with the OSM antagonizing B-K5 antibody, the BP-8 virtually did not affect the binding of CNTF on its receptor (Table I), indicating that its blocking activity is likely mediated at a downstream level.

Effect of anti-gp130 mAbs on Tyrosine Phosphorylation of the Transducing Protein—Because one of the earliest events observed after ligand binding on gp130 sharing receptors is a tyrosine phosphorylation of the transducing protein induced by the Jak kinase family (21, 22), we did study the effect of selected antibodies on gp130 signaling. Again, the SK-N-MC cell line was used to perform these experiments because it expressed all the different studied receptors and it was particularly easy to detect an induction of gp130 tyrosine phosphorylation in these cells. Fig. 5 shows that when the SK-N-MC cells were incubated in the presence of the selected antibodies alone at a concentration of 50 $\mu\text{g}/\text{ml}$, no induction of gp130 tyrosine phosphorylation could be observed, whereas 50 ng/ml of OSM led to a strong signal induction. Despite the fact that B-R3-mAb did not interfere with the binding of the ligands to their receptors (with the exception of OSM), B-R3 strongly antagonized gp130 activation independently of the nature of the cytokine used as stimulator. In the same experiments, the B-T6 nonblocking antibody did not affect the observed responses. This result clearly indicates that the B-R3 antibody was mainly acting at the signaling level rather than on the interactions of the ligands with their respective high affinity receptors. In addition to an IL-6-induced homodimerization of

² K. Turner, personal communication and unpublished observation.

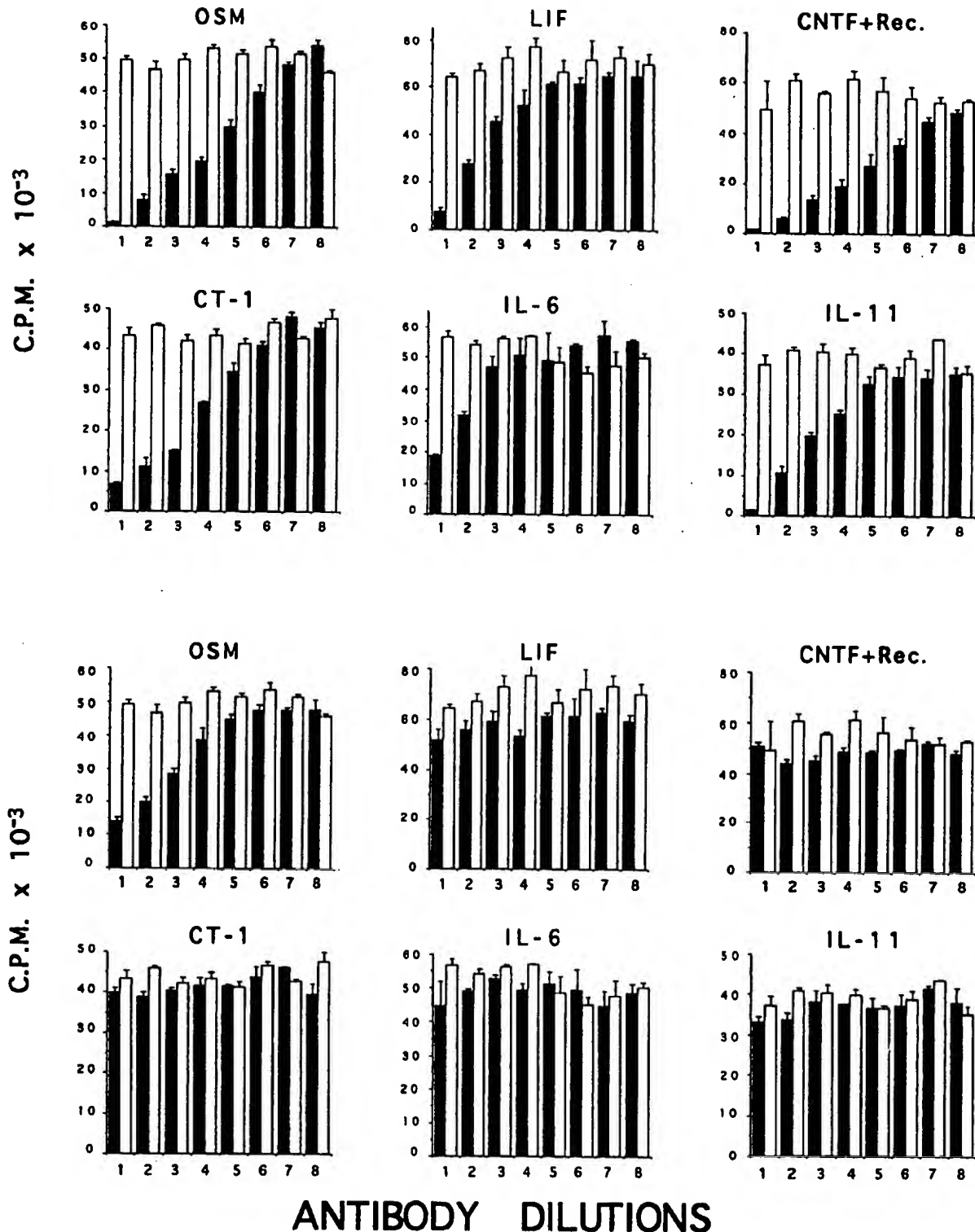


FIG. 2. Neutralizing activity of gp130 antibodies in the TF1 cell line proliferative assay. 15×10^3 TF1 cells were incubated in the presence of a fixed concentration of IL-6 (20 ng/ml), IL-11 (100 ng/ml), OSM (10 ng/ml), LIF (50 ng/ml), CT-1 (5 ng/ml), or CNTF (20 ng/ml) plus soluble CNTF receptor (100 ng/ml). 5-fold dilutions of B-R3 antibody (black bars in A), B-K5 antibody (black bars in B), or control IgG antibody (white bars in A and B) were added in triplicate. The first dilution of antibody corresponded to a final concentration of 50 μ g/ml in the assay. After a 72-h culture period, 3 HTdr was added for 4 h, and the incorporated radioactivity was determined.

gp130, the transducing protein could also heterodimerize with gp190/LIF receptor after LIF, OSM, CT-1, or CNTF activation (9, 14, 16). To determine whether B-R3 could antagonize gp130 association with gp190/LIF receptor, both subunits were copre-

cipitated in the presence of Brij 96 detergent, and the phosphorylation level of this latest subunit was analyzed after LIF activation (Fig. 6). Similar to the result obtained for gp130, the phosphorylation level of gp190 was dramatically reduced in the

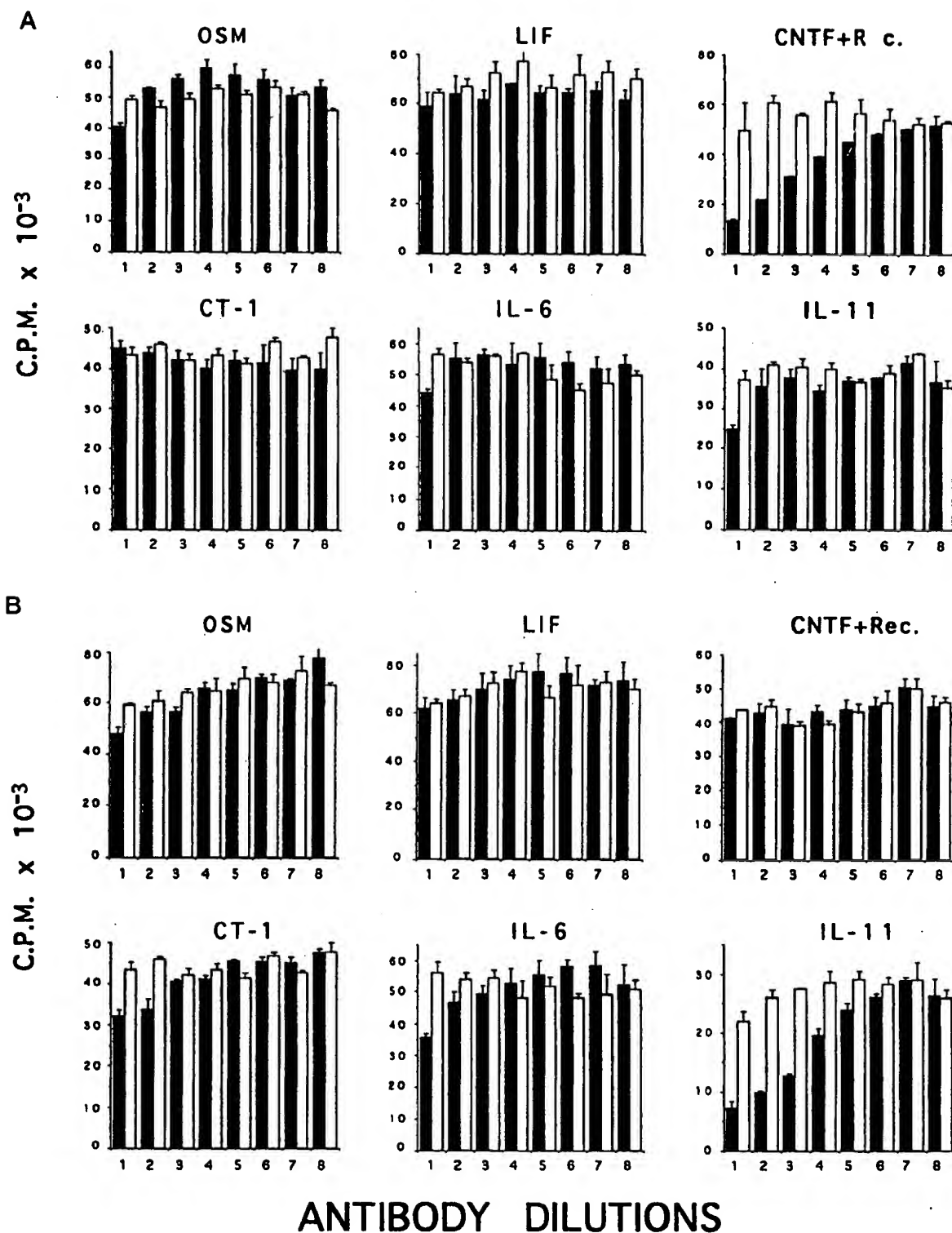


FIG. 3. Neutralizing activity of gp130 antibodies in the TF1 cell line proliferative assay. 15×10^3 TF1 cells were incubated in the presence of a fixed concentration of IL-6 (20 ng/ml), IL-11 (100 ng/ml), OSM (10 ng/ml), LIF (50 ng/ml), CT-1 (5 ng/ml), or CNTF (20 ng/ml) plus soluble CNTF receptor (100 ng/ml). 5-fold dilutions of B-P8 antibody (black bars in A), B-P4 antibody (black bars in B), or control IgG antibody (white bars in A and B) were added in triplicate. The first dilution of antibody corresponded to a final concentration of 50 μ g/ml in the assay. After a 72-h culture period, 3 HTdr was added for 4 h, and the incorporated radioactivity was determined.

presence of the B-R3 antibody. Moreover, B-R3 mAb abrogated the gp130/gp190 association, and the latest chain did not co-precipitate with gp130 under these conditions. An irrelevant IgG2a antibody did not affect the observed response. These

results strongly supported the notion that B-R3 inhibited the homo- or heterodimerization of the transducing receptor subunits.

In addition to the inhibition of phosphorylation observed in

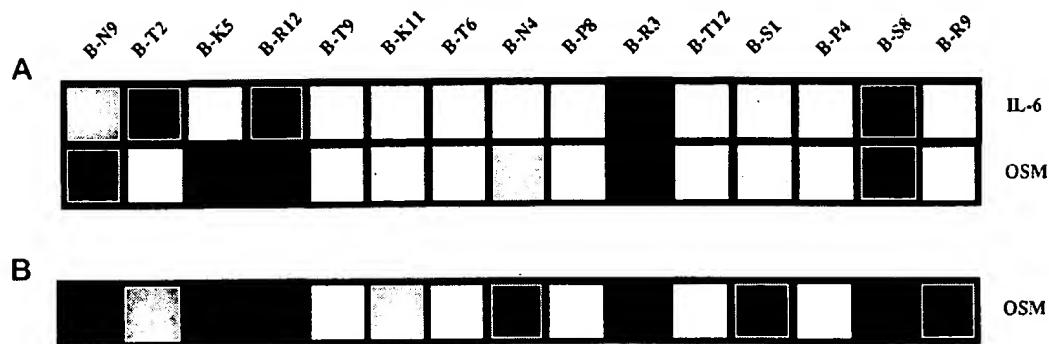


FIG. 4. A, inhibition of haptoglobin secretion by anti-gp130 antibodies in HepG2 cell line. HepG2 cells were plated in triplicate in the presence of 10 ng/ml IL-6 or 20 ng/ml OSM and 5-fold dilutions of antibodies (highest concentration of antibody was 50 μ g/ml). After 48 h the haptoglobin content of the culture supernatants was determined by ELISA. The figure summarizes a 50% reduction of haptoglobin secretion observed for an antibody concentration of 50 μ g/ml (light gray), 2 μ g/ml (dark gray), 80 pg/ml (black), or the absence of neutralizing effect with 50 μ g/ml of antibody in the culture (white). B, protective effect of gp130 mAbs on OSM induced lysis of the A375 melanoma cell line. Cells (3×10^3) were plated in the presence OSM (20 ng/ml) and serial dilutions of anti-gp130 mAbs for a 96-h culture. 3 HTdr was added to each well for the last 4 h of the culture, and the incorporated radioactivity was determined. B represents a 50% protective effect of each antibody for a concentration of 50 μ g/ml (light gray), 10 μ g/ml (dark gray), 2 μ g/ml (black) or the absence of protective effect with 50 μ g/ml of antibody (white).

TABLE I
Interactions of anti-gp130 mAbs on the binding of the IL-6 type cytokines to their receptors

SK-N-MC cells were incubated in the presence of 666 nM studied antibodies for 1 h. Serial dilutions (starting from 1 nM) of the indicated radiolabeled cytokines were added for an additional 2-h incubation period at 4 $^{\circ}$ C (final antibody concentration, 333 nM). Nonspecific binding was evaluated by including a 100-fold excess of unlabeled cytokine. Determination of high affinity binding components and site number expressed per cell were performed according to Scatchard.

mAb	IL-6		OSM		LIF		CNTF	
	K_d	Sites/cell	K_d	Sites/cell	K_d	Sites/cell	K_d	Sites/cell
O	189	452	203	508	243	221	42	253
B-R3	851	430	no signal		307	212	100	209
B-K5	354	563	no signal		186	221	39	235
B-P8	591	526	ND		177	273	34	205
B-P4	406	447	49	491	204	246	53	266
B-T6	203	472	160	486	175	246	47	313

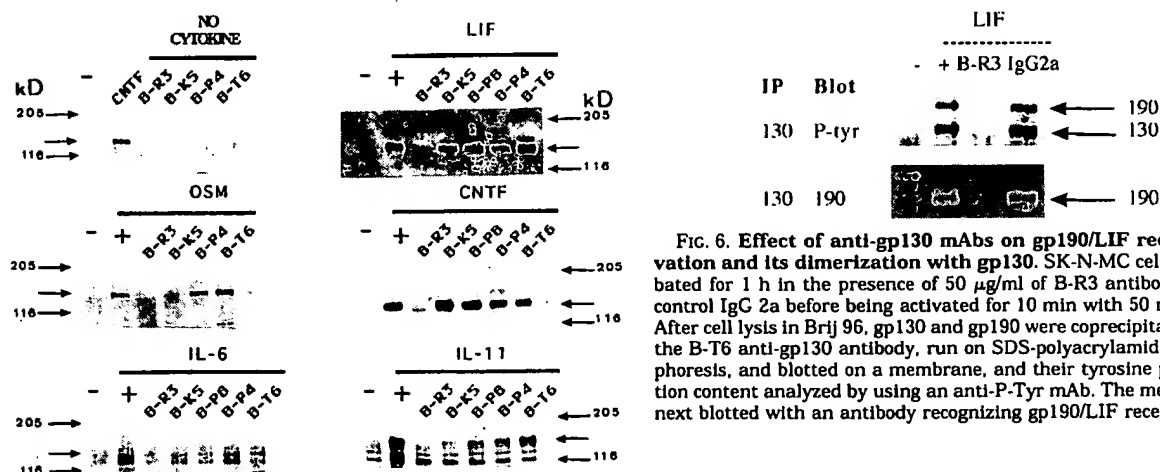


FIG. 5. Effect of anti-gp130 mAbs on tyrosine phosphorylation of gp130 receptor transducer. SK-N-MC cells were incubated for 1 h in the presence of 50 μ g/ml of the indicated antibodies before being activated for 10 min with the IL-6-related cytokines added at a concentration of 50 ng/ml. After cell lysis, gp130 was immunoprecipitated, run on SDS-polyacrylamide gel electrophoresis, and blotted on a membrane and its tyrosine phosphorylation content was analyzed by using an anti-P-Tyr mAb. The position of gp130 is indicated by an arrow.

FIG. 6. Effect of anti-gp130 mAbs on gp190/LIF receptor activation and its dimerization with gp130. SK-N-MC cells were incubated for 1 h in the presence of 50 μ g/ml of B-R3 antibody or with a control IgG 2a before being activated for 10 min with 50 ng/ml of LIF. After cell lysis in Brij 96, gp130 and gp190 were coprecipitated by using the B-T6 anti-gp130 antibody, run on SDS-polyacrylamide gel electrophoresis, and blotted on a membrane, and their tyrosine phosphorylation content analyzed by using an anti-P-Tyr mAb. The membrane was next blotted with an antibody recognizing gp190/LIF receptor.

observed in the presence of OSM. B-K5 mAb was without effect or with very marginal effects on the other detected responses (Fig. 5). This is in line with the fact that B-K5 recognized an important site of gp130 implicated in OSM binding. In contrast to the situations observed for the B-R3 and B-K5 antibodies, the phosphorylation level of gp130 induced by IL-11 or CNTF was not affected by the respective addition of B-P4 or B-P8 mAbs in the experiments (Fig. 5), whereas a similar concentration of these two antibodies entirely blocked the proliferation of the TF1 cell line to the same ligands. These results suggest that a global analysis of gp130 phosphorylation level might not be sufficient to discern subtle regulations of the activation processes or that additional events independent from tyrosine

the presence of the B-R3 antibody, analysis of gp130 immunoprecipitated cell extracts obtained after incubating the cells with the B-K5 mAb and cytokines led to the conclusion that this latest antibody specifically blocked activation of gp130

phosphorylation could be essential to regulate the gp130-mediated proliferation.

DISCUSSION

The existence of shared receptor transducing proteins was reported for several families of cytokine receptors (35). The recruitment of common receptor structures by different cytokines explain in part the observed redundancy of their biological properties. This is true for the shared use of the γ chain of the IL-2 receptor by the IL-2 (36), IL-4 (37), IL-7 (38), IL-9 (39), and IL-15 (40) receptors. Recent reports have demonstrated that the β chain of the IL-2 receptor was also implicated in the formation of the IL-15 receptor (40) and that the IL-4 receptor binding component was a part of IL-13 receptor, as well (41). In addition to the T/B cell growth factor receptors, the receptors for the myeloid lineage acting cytokines, IL-3, IL-5, and GM-CSF, were particularly well analyzed. The studies have reported that these receptors were composed of specific α binding components for IL-3, IL-5, or GM-CSF which, associate to a common β chain element to generate high affinity receptors (42–45). As mentioned in the introduction part, gp130 transducing protein is able to homodimerize following IL-6 binding (and possibly IL-11) or to heterodimerize with gp190/LIF receptor after activation by LIF, CNTF, CT-1, and OSM (9, 14, 16). Regarding to OSM receptor another not yet characterized transducing protein can associate to gp130 and constitutes the type II OSM receptor (19, 25).

The activation processes of sharing subunit receptors by one designed cytokine led to many unresolved questions. Among them is a common transducing receptor activated through a unique activation site, or does it involve different domains, depending on the nature of the recruiting cytokine? The answer to this question could be important for the design of specific antagonisms.

In the present study we have analyzed a series of newly derived monoclonal antibodies raised against gp130 transducing component and got a partial response to this question. Among the studied antibodies four of them were analyzed in detail. The inhibiting B-R3 antibody was able to block the responses to all the IL-6 cytokine family. With the exception of OSM, B-R3 only mildly interfered with the binding of the cytokines to their receptors. Analyses of the gp130 phosphorylation patterns indicated that B-R3 was acting at the dimerization level of the transducing subunits and was able to inhibit the heterodimerization and the association between gp130 and gp190 in the presence of LIF. We recently did identify the epitope recognized by B-R3 mAb by using a peptide phage display library. DNA sequence analyses defined an amino acid motif sharing some features with regions previously identified to be important for the dimerization of erythropoietin or growth hormone receptors (46, 47), supporting the notion that B-R3 inhibits the multimerization of the signal transducing subunits.³ A parallel can be made with the TUGm2 antibody directed against the γ subunit of IL-2 receptor, which was found to inhibit the proliferative response to IL-2, IL-4, IL-7, IL-9, and the heterodimerization of the γ chain with the respective binding subunits, but which only slightly decreased by a 3–6-fold the affinity of radiolabeled IL-2, IL-4, and IL-7 to their receptors and did not affect the IL-9 binding (36–39).

In addition to the pan-blocking antibody group, we did identify antibodies able to specifically interfere with a single member of this cytokine family. This is particularly true for the responses mediated after activation by OSM, CNTF, or IL-11. Regarding LIF or CT-1, we did not identify antibodies able to antagonize these cytokines in a specific manner. This could be

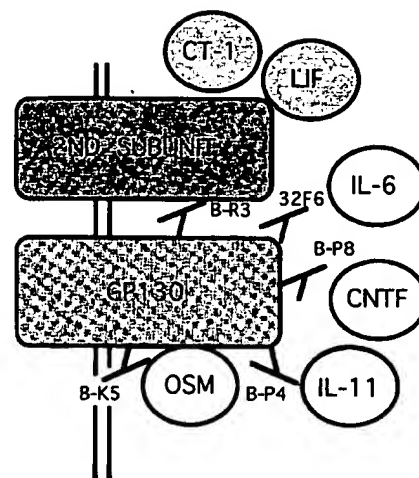


FIG. 7. Schematic representation of the gp130 activation sites defined by monoclonal antibodies.

linked to the fact that both LIF and CT-1 preferentially bind to gp190/LIF receptor, and gp130 acts more like a converter protein rather than a real binding subunit (9, 13). In agreement with this observation, we can see that the antibodies abrogating LIF responses belong to the same group as B-R3. For CT-1 two additional antibodies (B-T2, B-R9) with a broad blocking spectrum also neutralized the cytokine response.

Regarding to the IL-6 response, we very recently identified one additional antibody, 32F6, able to block very specifically the IL-6 response without affecting the activation processes mediated by any other member of the IL-6 family.⁴ To summarize the performed observations we propose a model (Fig. 7) where the defined activation sites of gp130 are represented.

Interestingly the use of antibodies directed against the β IL-2 receptor led to the isolation of mAbs able to neutralize the IL-15 response without affecting the IL-2 triggered proliferation, showing that in this receptor model different functional epitopes were also expressed by the common β IL-2 receptor chain (40). Similarly, antibodies generated against the shared transducing β subunit of the IL-3, IL-5, and GM-CSF receptors were shown to antagonize solely one ligand (48). This work was recently reinforced by site-directed mutagenesis analysis of the external part of the β subunit of human GM-CSF, IL-3, and IL-5 receptors. It was shown that alanine substitution of the residues 365–368 abolished GM-CSF and IL-5 high affinity binding but only marginally affected IL-3 high affinity binding, indicating that different regions of the β transducer molecule were required in function of the recruiting cytokine (49).

Analysis of the biological properties of the antibodies able to specifically neutralize OSM, CNTF, or IL-11 led to the conclusions that several different processes could antagonize specifically one given response. In the case of the B-K5 antibody, the abrogation of the OSM response mediated through type I or type II receptor was linked to an inhibition of OSM binding to gp130. Similar results were obtained in the hematopoietic BAF/Bo3 cell line stably transfected with a cDNA encoding for human gp130.⁵ B-P8 and B-P4 antibodies were able to mutually displace each other in cytofluorometry; nevertheless the use of bioassays revealed that they likely recognized closed but different epitopes respectively implicated in CNTF and IL-11 recruitment of gp130. Tyrosine phosphorylation studies performed in the presence of CNTF or IL-11 and their cognate

⁴ S. Chevalier, unpublished results.

⁵ S. Chevalier, M. Fourcin, O. Robledo, J. Wijdenes, A. Pouplard-Barthelax, and Hugues Gascan, unpublished results.

³ P. Auguste, manuscript in preparation.

antibodies revealed that they failed to inhibit gp130 activation. To try to elucidate this apparent contradiction, gp130 phosphorylation analyses were conducted in HepG2 and TF1 cell lines. The results observed in HepG2 cells were identical to those presented in Fig. 6 for the SK-N-MC cell line (data not shown). Regarding the TF1 cell line the use of 10^9 cells per condition led to a subluminal signal after IL-11 or CNTF treatment as we previously reported (28, 32), whereas for the SK-N-MC cell line (or HepG2 cells) $1-3 \times 10^6$ cells allowed a clear detection of the induction of gp130 phosphorylation. These results suggested that gp130 phosphorylation was not an essential element to induce a proliferative response in the TF1 line. This is in agreement with observations performed with intracellular truncated forms of gp130 transfected in the BAF/Bo3 cell line and showing that tyrosine phosphorylation of a $\Delta 65$ gp130 truncated mutant was not required for proliferation (50). Similar results were recently published regarding to the erythropoietin receptor, where the removal of all tyrosine phosphorylation sites from this receptor still allowed a proliferative response to the cytokine (51). On the other hand it has been clearly established that the tyrosine phosphorylation sites of the distal parts of gp130 or gp190, which defined a motif known as box 3, were necessary to activate STAT3 and the subsequent transcription events (22). In the light of these reports we hypothesized that the B-P8 or the B-P4 antibodies could neutralize the proliferative responses to CNTF or IL-11 without affecting the gp130 tyrosine phosphorylation/STAT3 transcription pathway (22-24). We have undertaken to study the influence of B-P8 and B-P4 antibodies on the JAK/STAT pathways, and preliminary results indicated that these antibodies affected only some elements of the Jak/STAT transduction cascade. One interpretation could be that the B-P8 and B-P4 mAbs lowered the interaction strengths between the transduction receptor components leading to an incomplete response, sufficient to induce gp130 phosphorylation but not DNA synthesis and proliferation. In line with the obtained results, it has recently been demonstrated that it was possible to dissociate the IL-6 response element STAT3-dependent transcriptional control from hematopoietin response element pathway (23). The former was dependent from the box 3 phosphorylation mediated by STAT3, whereas the latest one was box 3-independent. Similarly it was also reported the possibility to alter T cell receptor activation with peptides or antigens and to convert fully activated complexes into partially activated T cell receptors leading to different phosphorylation patterns and responses (52, 53).

In conclusion, as it appears in Fig. 7, we have shown that the cytokines belonging to the IL-6 family that preferentially bind to gp130 (IL-6, IL-11, OSM, and CNTF) recruited the transducing protein through distinct functional motifs. In addition, the B-R3 antibody recognized an epitope that was important for gp130 multimerization and signaling. Moreover, additional contacting points between the members of the IL-6 cytokine family and gp130 probably exist, as suggested for example by the observed pattern of the B-T2 mAb, which neutralized all responses except those of LIF and OSM.

Several cytokines of this family have been reported to be implicated in pathologies. This is the case for IL-6 in multiple myeloma (54), IL-11 was found to be an autocrine growth factor for some megakaryoblastic leukemia cell lines (55), and OSM is a progression factor in Kaposi's sarcoma (56). The availability of specific inhibitors for one single cytokine opens interesting possibilities for the clinical application of some of the described antibodies.

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